Spread of *Penicillium digitatum* and *Penicillium italicum* During Contact Between Citrus Fruits

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ABSTRACT

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Postharvest infection of healthy citrus fruit by *Penicillium digitatum* (the cause of green mold) and *P. italicum* (the cause of blue mold) in contact with decaying fruit was influenced by the amount of aerial mycelium produced on the surface of the lesion at the point of contact with the healthy fruit and the location of the injury-infection site in relation to the contact point. The establishment of infection courts in healthy fruit by both organisms was attributed to injury of healthy rind by an acidic excretion, principally galacturonic acid, from the decay lesions. Treatment of intact fruit rind with spores of either organism contained in a nutrient solution resulted in infection only after the addition of galacturonic acid. *P. italicum* spread from decaying to healthy fruit more frequently than did *P. digitatum* when contact between the infected and uninfected fruit resulted from enlargement of the lesion. *P. italicum* produced sparse aerial mycelium at the contact point, which did not prevent injury of the healthy rind by the excretion. In contrast, *P. digitatum* produced a thick mat of aerial mycelium at the contact point, which prevented the acidic excretion and thus prevented rind injury and infection. However, when formation of aerial mycelium at the contact point was prevented by the presence of moisture, *P. digitatum* also spread to healthy fruit. Both organisms spread when the injury-infection site was in contact with the healthy fruit. The exudate from the injury prevented development of aerial mycelia, thus exposing the healthy rind to the injurious acidic excretion.

Green and blue mold caused by *P. digitatum* Sacc. and *P. italicum* Wehmer., respectively, are two important postharvest diseases of citrus fruit. Both organisms initiate infection via a rind injury, usually into the mesocarp, and produce a soft rot (5,10). However, *P. italicum* forms lesions less rapidly (6) and spreads to healthy fruit by contact more frequently than does *P. digitatum* (8). Fruits affected by blue mold often are found in clusters, whereas those with green mold usually occur singly or in pairs (7). Organic acids are important in overcoming resistance of uninjured citrus rind to infection by *Penicillium* spp. and may cause tissue softening and decreased cell turgor (10). Accumulation of galacturonic acid occurs in tissue infected by *Penicillium* spp. (2,3,11) due to the activity of polygalacturonases (PG) (2–4).

The objective of this study was to determine the various factors involved in the spread of *P. digitatum* and *P. italicum* during contact between diseased and healthy fruit.

MATERIALS AND METHODS

Inoculations. Mature oranges (*Citrus sinensis* (L.) Osbeck 'Valencia') were washed and surface sterilized with 1% sodium hypochlorite as previously described (3). Inoculations were made by puncturing the rind with a dissecting needle wetted with spores of either *P. digitatum* or *P. italicum* suspended in distilled water containing 0.01% Triton X-100. Inoculated fruits were held at 24 C and near 100% relative humidity (RH) for the development of decay. Rate of lesion development was based on observations of 10 fruits.

Organic acid analysis. Organic acids in the decayed rind were extracted and analyzed by a modified procedure of Fernandez-Flores et al (8). Decayed peel was homogenized in water and centrifuged. The supernatant was adjusted to 80% ethanol and centrifuged. One milliliter of saturated lead acetate solution was added to 10 ml of the supernatant, and the precipitate containing the organic acids was collected by centrifugation. The organic acid precipitate was serially washed with ethanol, acetone, diethyl ether, and then vacuum dried. Organic acids were silylated with Tri-Sil (Pierce Chemical Co., Rockford, IL 61105) and analyzed with a Hewlett-Packard Model 5730A gas chromatograph equipped with a flame ionization detector and an SE 30 column and programmed for a temperature rise of 4 C/min from 70 to 210 C.

Diffusion of the organic acids through the intact surface of the lesion was determined by inverting 13-mm-diameter plastic vials containing 2 ml of distilled water on the surface of the water-soaked area at the edge of the lesion and where the decayed area had contacted the healthy fruit. The organic acid content in the vials was determined at 24 hr by using the titration procedures of Sinclair and Eny (12).

Polygalacturonase analysis. Presence of PG activity in the exudate at the injury-infection site and in the exudate on surfaces of healthy fruits at the contact point was determined by the viscosity reduction method (3). The exudate at the contact point was collected by rinsing the area with 2 ml of water.

Infection. Spread of decay from an infected to an uninfected fruit by contact was studied by supporting an inoculated fruit on top of a sound fruit in a beaker. Fruit were washed on a commercial fresh fruit washer equipped with 17 tumbler brushes rotating at 200 rpm. Areas free of surface blemishes were selected as contact points. The injury-infection site for both organisms was placed either in contact with the uninfected fruit or positioned at a right angle to the contact point (Table 1). An additional series of *P. digitatum*-inoculated and healthy fruit, with the injury-infection sites at right angles to the contact point, were held keeping the contact point moist by

TABLE 1. Decay of healthy fruit caused by contact with the injury-infection site or lesion of fruit infected by *Penicillium digitatum* or *P. italicum*

Contact site[a]		Decay (%)[b]
	P. digitatum	82
	P. italicum	87
	P. digitatum	16
	P. italicum	78
	*P. digitatum*[c] (Contact point wet)	100

[a] Arrow indicates injury-infection site.
[b] After 7 days at 23 C. Each treatment consisted of five replications of 10 fruit each.
[c] Surface of lesion at contact point was moist and free of aerial mycelium.

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periodically wetting a piece of cheesecloth placed between the fruit. All fruits were kept at 23 C and 92% RH.

The effect of galacturonic acid on aiding infection was studied by placing on the intact rind the spores of either organism suspended in 2 ml of 50 mM galacturonic acid in 2.4% Difco potato-dextrose broth (PDB). The spore suspensions were kept in continual contact with the rind for 6 days with the aid of inverted plastic vials 13 mm in diameter. Fruits were evaluated for decay during this 6-day period. Fruits were washed either by hand with cheesecloth or with the commercial washer.

Histology. Rind at the point of contact of infected and healthy fruit was fixed in 3% glutaraldehyde in phosphate buffer (pH 7),

TABLE 2. Effect of galacturonic acid in nutrient solution on infection of uninjured Valencia orange rind by *Penicillium digitatum* and *P. italicum*

Galacturonic acid[a] (mM)	Infection (%)[b]	
	P. digitatum	*P. italicum*
Hand washed[c]		
0	0	0
50	17	30
Machine washed[d]		
0	7	0
50	93	87

[a] Added to potato-dextrose broth containing 10^6 spores per milliliter.
[b] After 6 days at 23 C and 92% RH.
[c] Washed by hand with cheesecloth.
[d] Washed with a commercial washer.

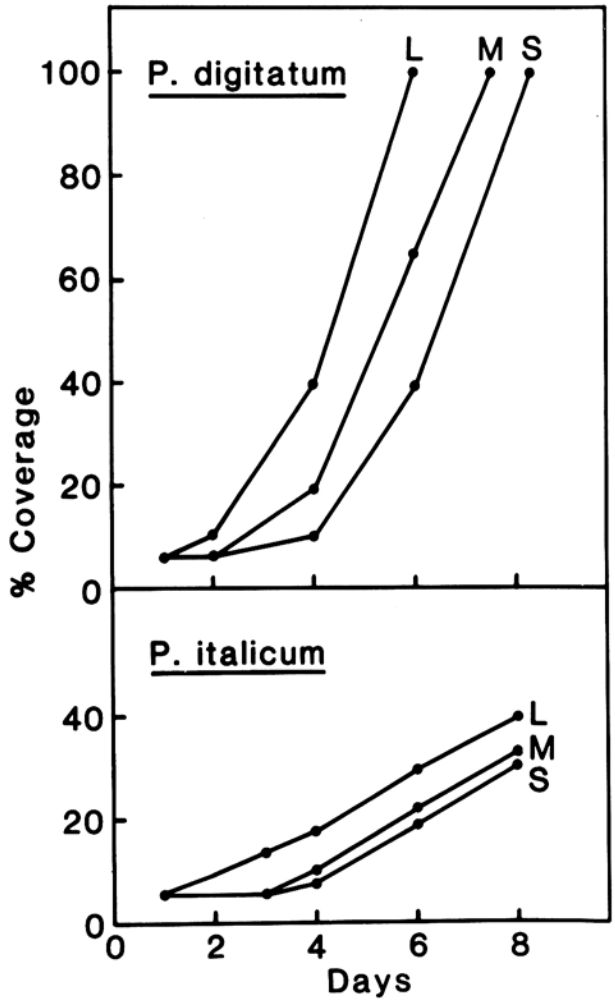

Fig. 1. Rate of development of decay caused on Valencia orange fruits by *Penicillium digitatum* and *P. italicum*. L = lesion, M = surface mycelium, and S = sporulating mycelium.

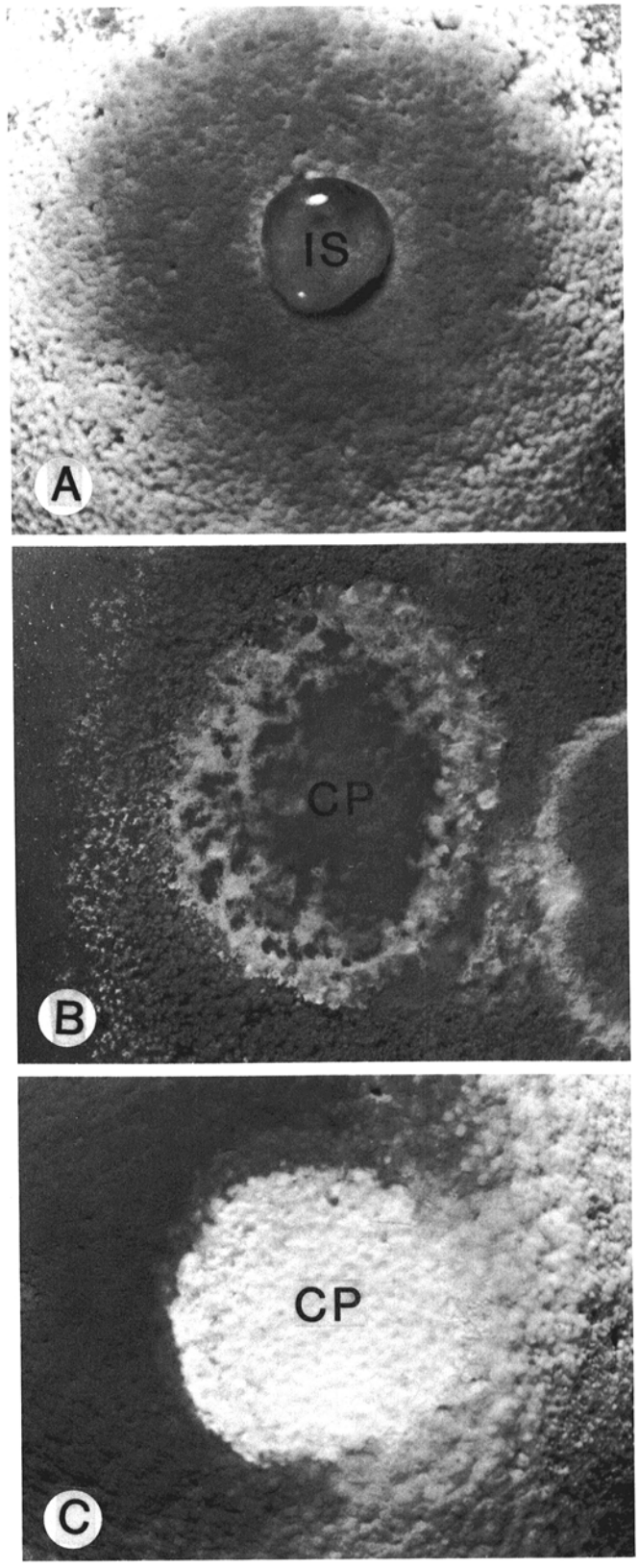

Fig. 2. Valencia orange fruit rind infected with *Pencillium digitatum* or *P. italicum*. **A,** Fruit infected with *P. digitatum* showing exudate at injury-infection site. **B,** Contact point of a fruit infected with *P. italicum* showing the relative lack of surface mycelium. **C,** Contact point of a fruit infected with *P. digitatum* showing the area covered with white nonsporulating mycelium. IS = infection site, CP = contact point.

dehydrated with tertiary-butyl alcohol, and embedded in paraffin. Sections, 10 μm thick, were made with a Sorvall JB-4 microtome (DuPont Co., Newtown, CT 06470) and stained with aniline blue.

Fruit surfaces in three areas of the lesions formed by *P. digitatum* and *P. italicum* were examined with a JEOL-JSM35 scanning electron microscope (JEOL USA, Inc., Medford, MA 02155). Tissue sections, 3 × 3 mm, were taken from lesions where the mycelium was rupturing the rind, from the water-soaked margin at the edge of the lesion, and from outside the lesion in an unaffected area of the same fruit. All samples were dewaxed with chloroform at 58 C (1,9) and fixed in 3% glutaraldehyde in 0.02 M potassium phosphate buffer, pH 7, at 23 C for 7 hr. They were washed in buffer, postfixed in 2% osmium tetroxide overnight at 10 C, dehydrated in an acetone/water series and then in a Freon TF/acetone series, and critical-point dried in a Bomar 950 dryer (Bomar, Inc., Tacoma, WA 98401). After being mounted on aluminum stubs, the samples were coated with 10^{-2} μm of gold-palladium (60:40) on a Technic sputter coater (Technic EMS, Springfield, VA 22153) and observed with the scanning electron microscope operated at 25 kV and 100–110 μA.

RESULTS

The rate of decay development caused by *P. digitatum* and *P. italicum* on a citrus fruit is shown in Fig. 1. *P. digitatum* decayed the entire fruit in 6 days, whereas *P. italicum* decayed only 40% of the fruit after 8 days. Development of the water-soaked area was followed in 24–36 hr with coverage by aerial mycelia.

Tissues infected with *P. digitatum* contained 15.6 mg of organic

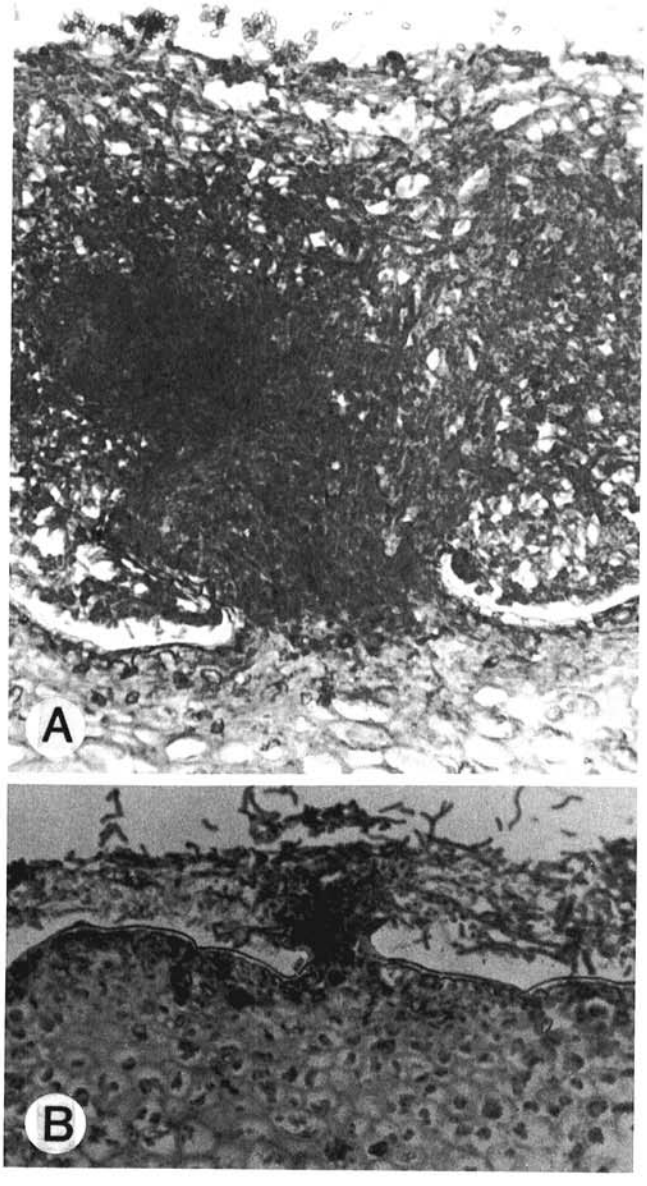

Fig. 3. Cross section of the rind of a decayed Valencia orange fruit showing aerial mycelium that developed at its point of contact with a healthy fruit. Decay was caused by **A,** *Penicillium digitatum* and **B,** *P. italicum* (both ×160).

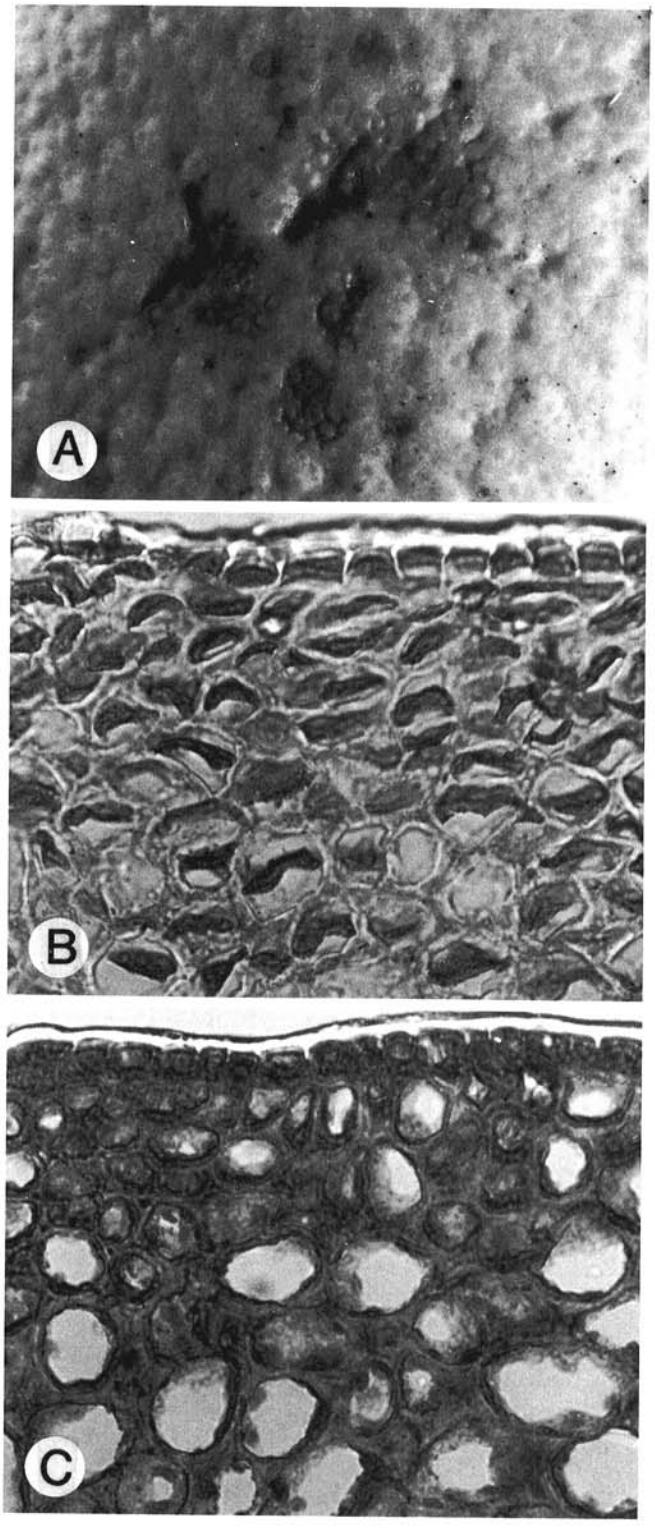

Fig. 4. Cross sections of Valencia orange rind. **A,** Pitted rind of a healthy fruit at its point of contact with a fruit infected with *Penicillium italicum*. **B,** Pitted rind showing collapsed cells (×500) and **C,** Normal, uninjured rind (×500).

acids per gram (fr wt) of tissue, whereas tissue infected with *P. italicum* contained 11.0 mg/g. Galacturonic acid was the primary acid, comprising 12.5 mg/g and 8.6 mg/g in tissue decayed by *P. digitatum* and *P. italicum*, respectively. Citric, malic, and succinic acids comprised 20–22% of the total organic acids. The organic acids in the decayed tissue readily diffused through the water-soaked surface of both lesions; 31.7 ± 6.2 and 17.6 ± 2.9 microequivalents of organic acids per square centimeter of (μeq/cm^2) surface area in 24 hr for *P. digitatum* and *P. italicum*, respectively. However, diffusion of organic acids through the mycelial mat of *P. digitatum* on the infected fruit at the point of contact was only 1.9 ± 0.9 μeq/cm^2 of surface area after 24 hr. The

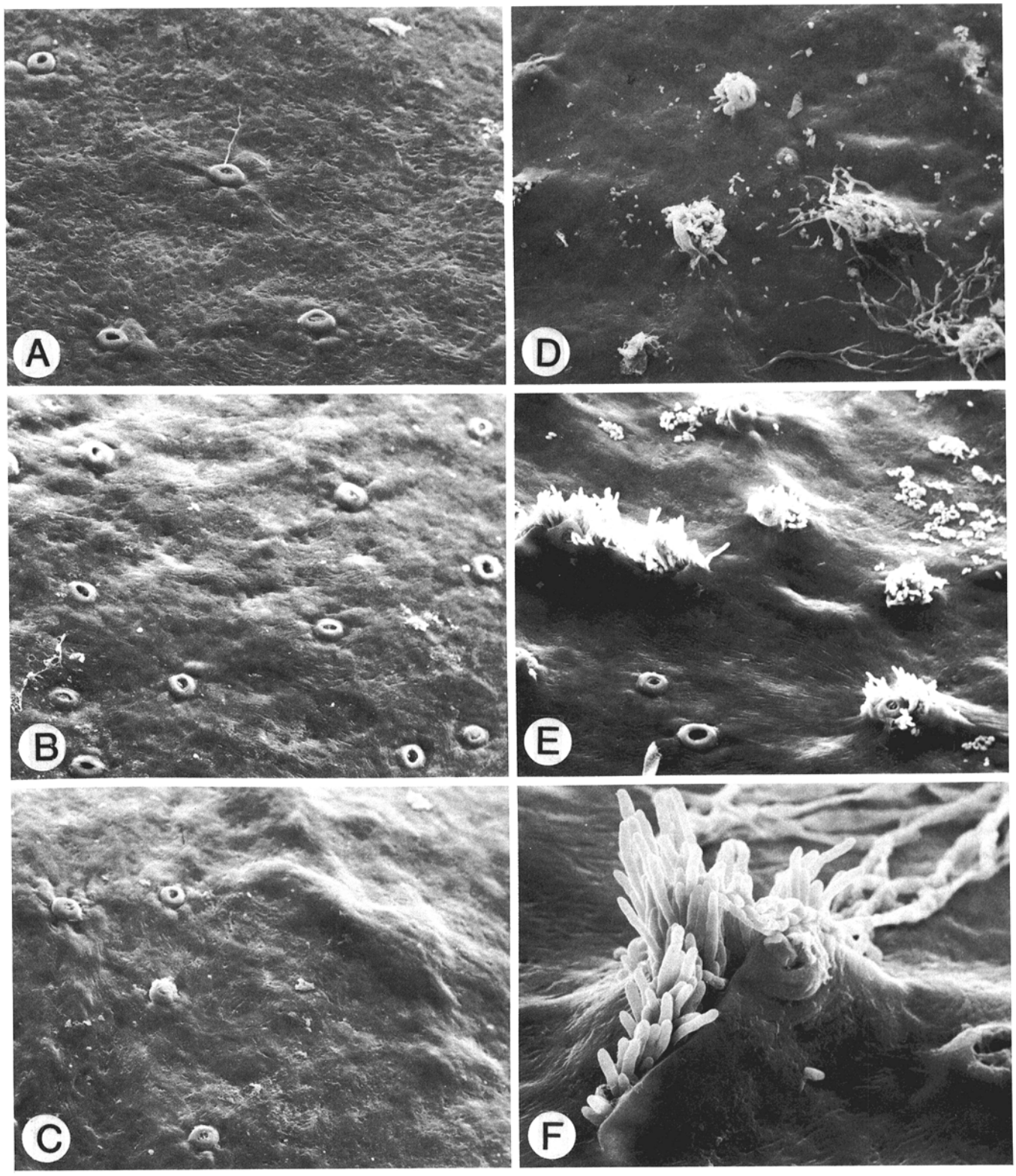

Fig. 5. Scanning electron micrographs of the dewaxed rind surfaces of infected and healthy Valencia oranges. **A,** Uninfected fruit surface (×200). **B,** Water-soaked area near the outer edge of a lesion formed by *Penicillium italicum* (×200). **C,** Water-soaked area at the edge of a lesion formed by *P. digitatum* (×200). **D,** Area within the lesion showing hyphae of *P. italicum* emerging through the rind surface (×200). **E,** Hyphae of *P. digitatum* rupturing the epidermis (×200). **F,** Hyphae of *P. digitatum* emerging at a stoma (×600).

organic acid that diffused through the lesion at the point of contact of the fruit infected by *P. italicum* was 54.8 ± 8.3 μeq/cm^2 after 24 hr. The diffusates from both decays contained PG activity.

Both fungi spread from decayed to healthy fruit when the injury-infection site was in direct contact with the healthy fruit. When contact with healthy fruit was made by the advancing lesion, *P. italicum* spread more frequently than *P. digitatum* (Table 1). Spread of *P. digitatum* through contact by lesion enlargement was induced only when the contact point was continually wetted during lesion formation (Table 1).

Infection of intact rind by spores of *P. digitatum* and *P. italicum* in PDB was increased by the addition of galacturonic acid to the PDB solution (Table 2). Machine washing of the fruit markedly increased the amount of infection compared with hand-washing.

Development of the lesion at the injury-infection site was similar in the two decays. The surface of the rind immediately surrounding the injury site was relatively free of mycelium and it remained continually wet because of exudation from the decayed tissue (Fig. 2A). Where contact between the healthy fruit and lesion occurred by lesion enlargement, the surface of lesions formed by *P. italicum* was sparsely covered with aerial mycelium in the area of contact (Fig. 2B). In contrast, the surface of fruit infected with *P. digitatum* at the contact area was covered with nonsporulating aerial mycelium that was dry and free of diffusates (Fig. 2C). Cross sections of the surfaces of the decayed fruit at the point of contact showed that the thickness of the *P. digitatum* mycelial mat was ~385 μm (Fig. 3A) compared to 85 μm for mycelial mats formed by *P. italicum* (Fig. 3B). Where spread by either organism occurred, pits in the rind of the uninfected fruit indicated initial stages of infection (Fig. 4A). Sections taken from slightly pitted tissue did not contain hyphae, but cell contents had collapsed in affected tissue (Fig. 4B), but not in healthy tissue (Fig. 4C).

For scanning electron microscopy, rind surfaces were dewaxed to expose breaks in the epidermis. The lesion surfaces on fruit infected by both *P. digitatum* and *P. italicum* were similar. Epidermal breaks were not observed in healthy tissue (Fig. 5A) or in tissue from the water-soaked area near the edge of the lesion (Fig. 5B and C). Within the lesion, numerous breaks were present near filaments of aerial hyphae (Fig. 5D–F). These breaks were caused by hyphae emerging near the stomata (Fig. 5F).

DISCUSSION

The frequency of infection by *P. italicum* and *P. digitatum* was similar in healthy fruit placed in direct contact with the injury-infection site. Exudate discharged from the injury during development of the decay restricted development of aerial mycelium in the immediate vicinity of the injury, thus keeping the healthy rind in continual contact with the exudate. The major acid in the exudate was galacturonic acid, which was formed during pectin degradation by PG produced by these fungi (2–4). Continual contact of the healthy fruit rind with the acidic exudate eventually caused pitting of the exocarp and permitted hyphal penetration. The principal factor causing injury to the epicarp is attributed to the phytotoxicity of the exuded organic acids. Treatment of healthy rind with a spore suspension in PDB did not cause infection even though the spores germinated and the resulting hyphae grew. Infection occurred only after the addition of galacturonic acid to the PDB-spore suspension. Interestingly, the effectiveness of galacturonic acid in predisposing the rind to infection was dependent on machine washing of the fruit. Apparently the vigorous brushing action damages the cuticle and allows the galacturonic acid to penetrate the healthy tissue. The role of PG in the exudate is considered to be secondary, causing tissue softening and aiding hyphal penetration after the initial injury has occurred.

The difference between the two fungi in the frequency of spread from decayed to healthy fruit as a result of the advancing lesion is related to the amount of aerial mycelium formed on the surface of the infected fruit at point of contact. *P. digitatum* formed a thick mat of mycelium on the surface of the infected fruit within 24–36 hr after the edge of the developing lesion had touched the adjacent healthy fruit. In contrast, *P. italicum* usually formed a sparse layer of mycelium on the surface. The heavy mycelial mat produced by *P. digitatum* prevented the acidic exudate from contacting the healthy rind, thus preventing rind injury and infection. The mycelial mat produced by *P. italicum* was too sparse to protect the rind of healthy fruit from the action of the excreted acidic medium. Emergence of hyphae through the cuticle in the area of contact increased the diffusion of acids, thus enhancing injury to the healthy fruit rind.

Why the thick, nonsporulating mat of mycelium is produced at the point of contact by *P. digitatum* but not by *P. italicum* is not known. The lesion surfaces of both decays were similar. Organic acids within the decayed tissues readily diffused through the intact epidermis and cuticle of both lesions. *P. digitatum* can spread as rapidly as *P. italicum*. A high rate of contact infection occurred when formation of the aerial mycelium was prevented by moistening the contact point. Liquid on the fruit surface prevented development of the surface hyphae. Since *P. italicum* grows much more slowly than *P. digitatum*, adequate amounts of diffusates may accumulate at the decayed fruit surface in the contact area to prevent surface hyphae formation of *P. italicum*, but not of the more rapidly growing *P. digitatum*. We observed that the contact area of *P. italicum*-infected fruit consistently appeared moist after the lesion enveloped that area.

The differences in abilities of *P. digitatum* and *P. italicum* to develop aerial mycelium on diseased citrus fruit explain why no more than two contiguous fruits usually develop green mold, whereas a "nest" involving several fruits with blue mold is common in packed containers.

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